Genomic approaches to the initiation of DNA replication and chromatin structure reveal a complex relationship

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Abstract

The mechanisms regulating the coordinate activation of tens of thousands of replication origins in multicellular organisms remain poorly explored. Recent advances in genomics have provided valuable information about the sites at which DNA replication is initiated and the selection mechanisms of specific sites in both yeast and vertebrates. Studies in yeast have advanced to the point that it is now possible to develop convincing models for origin selection. A general model has emerged, but yeast data have also revealed an unsuspected diversity of strategies for origin positioning. We focus here on the ways in which chromatin structure may affect the formation of pre-replication complexes, a prerequisite for origin activation. We also discuss the need to exercise caution when trying to extrapolate yeast models directly to more complex vertebrate genomes.

Keywords: *DNA replication origin; nucleosome positioning; chromatin structure; transcription factors; genome-wide studies*

DNA replication must be tightly regulated to ensure cell survival and genome stability. The entire genome of the cell must be replicated once, and once only, to ensure the accurate transmission of the genetic information to successive generations of cells. DNA replication begins at specific positions in the genome called origins of replication. The pre-replication complex (Pre-RC) binds to these sequences: the origin recognition complex (ORC) binds to the origin, leading to the recruitment of the Cdc6 and Cdt1 proteins, followed by the putative helicase, the MCM complex [1]. This process is known as 'origin licensing' and occurs at the start of the G1 phase of the cell cycle. A recent study in budding yeast suggests that this process is highly dynamic in G1 [2]. This study shows that Pre-RCs can be displaced by transcription and reformed by continuous relicensing. Although the majority of replication origins in the genome of budding yeast are intergenic, a subset of Pre-RCs (35%) are formed at sites of synthesis of cryptic unstable transcripts (CUTs). In vertebrates, a transition known as the origin decision point (ODP) occurs during G1, fixing the position of the active Pre-RCs [3]. It is unknown whether this transition involves the selection of a subset of Pre-RCs from an excess of licensed starting points or whether Pre-RCs are dynamic during G1 as suggested in yeast. Either way, the final result is the establishment of a defined spatial program fixing the points at which DNA replication starts. Only licensed initiation sites can be used during S phase, and mechanisms preventing Pre-RC assembly during S phase prevent potentially dangerous re-replication. It has been clearly established, in various organisms, from yeasts to humans, that more origins are prepared for replication in G1 than are actually required during S phase (origin redundancy). This redundancy is a fail-safe mechanism, ensuring that replication restarts through the activation of 'dormant origins' when forks are arrested [4, 5]. The firing of origins in S phase depends on the co-ordinated action of kinases [6–8]. The phosphorylation of several initiation factors leads to unwinding of the DNA duplex at the origins and

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promotes the recruitment of the replisome. The principal actors and their stepwise association with chromatin have been described in detail [9]. However, it remains to be determined which steps are the most critical and how they are regulated. These issues are beginning to be resolved for budding yeast, thanks to the recent development of genome-wide studies. Investigations in the simple genome of this organism have revealed the existence of complex regulations and generated new hypotheses relating to origin selection in the genomes of complex vertebrates, which are 100 times larger.

The co-ordinate activation of replication origins operates at two levels. First, a spatial program regulates the positioning of replication origins within the genome. Secondly, a temporal program regulates the moment at which the various regions of the genome are replicated during S phase. In higher eukaryotes, replication timing domains are mega base-sized regions that result from the more or less synchronous firing of several origins. They may be replicated in early, mid or late S phase, with a continuous gradient between them. The recent development of high-throughput analyses has made it possible to study both the spatial and temporal programs at the genome-wide level [10–14]. Several good reviews discussing these aspects have recently been published [15–19]. We focus here on the recent results of these genome-wide studies on DNA replication, which have shed new light on the possible effects of chromatin structure on the regulation of DNA replication origins through Pre-RC assembly.

NUCLEOSOME-FREE REGIONS CONTROL ORIGIN SELECTION, FROM YEAST TO FLY

Genomic approaches have either focused on sites of Pre-RC assembly, through chromatin immunoprecipitation with antibodies against proteins of the Pre-RC, such as ORC subunits, or on the sites of origin activation during S phase. The first approach involves the mapping of potential origins of replication, whereas the second detects the final event in the cascade of origin activation, the formation of a replication bubble. Both approaches are instructive and comparisons of the results obtained with the two methods should provide clues to the regulated events occurring between Pre-RC formation and origin firing. Identification of licensed sites that do not fire under normal conditions would allow to distinguish a new class of Pre-RCs that could, for example, be used only in case of replication fork arrest. The mapping of replication origins in conditions of replicative stress would validate the hypothesis that these sites are indeed 'dormant origins'.

In *Saccharomyces cerevisiae*, the ORC binds the ARS (autonomously replicating sequence) consensus sequence (ACS), an 11 bp T-rich sequence that is necessary but not sufficient for origin activity [20]. The genome contains several thousand potential ACS sequence matches, only a few hundred of which are bound specifically by the ORC. A recent study in budding yeast refined previous maps of ORC binding sites by ChIP-seq techniques, thus providing a precise map of ORC-bound ACS (ORC-ACS)[21]. The aim was to determine what distinguishes ORC-ACS from non-replicative ACS (nr-ACS). Analyses of nucleosome positioning at ORC-ACS have revealed that the pattern of nucleosome occupancy at the ORC-ACS is similar to that of open promoters. It consists of a ~130 bp nucleosome-free region (NFR) (narrower than that for promoters) surrounded by −1 and +1 nucleosomes stably positioned and adjacent to a periodic series of nucleosomes. The absence of such precise nucleosome positioning at nr-ACS suggests that NFRs may be a hallmark of functional ACS. Attention then focused on the way in which such structural features are encoded. Nucleosome organization results from an interplay of multiple factors, including competition between site-specific DNA-binding proteins, chromatin remodellers and the DNA sequence preferences of nucleosomes themselves. The role of the primary DNA sequence was investigated by using a previously constructed map of *in vitro* assembled nucleosomes on the naked yeast genome to compare nucleosome positioning at ORC-ACS and nr-ACS [22]. This map revealed that the NFR induced by nr-ACS was much weaker than that induced by ORC-ACS, but precise periodic positioning of adjacent nucleosomes was not observed around ORC-ACS in the *in vitro* data. These findings confirm the observation that intrinsic nucleosome sequence preferences make a substantial contribution to nucleosome organization and chromatin function *in vivo*, and also demonstrate that many aspects of *in vivo* nucleosome organization are not explained by nucleosome sequence preferences. The authors therefore explored the possibility that additional *cis*-regulatory elements might contribute to origin function. They confirmed the previous

observation that there is a 4–6 bp island of A residues 50–100 bp downstream from the ACS, constituting a strong nucleosome-excluding signal specific to ORC-ACS. According to the final model for ORC-ACS selection, the establishment of large asymmetric NFRs would facilitate ORC binding, leading to the positioning of the +1 and −1 nucleosomes (Figure 1A). A similar study, with different, but largely overlapping ORC-ACS, reached analogous conclusions [23]. This study also showed that, when individual origins were analyzed, some origins, although efficient, lacked a clear NFR, suggesting that functional replication origins can be constructed with various nucleosome occupancy patterns and that an NFR is not necessary for origin specification.

A recent study in budding yeast also raised the question of origin diversity, by exploring the role of the conserved bromo-adjacent homology (BAH) domain of Orc1 in the selection of DNA replication sites within chromatin [24]. This ~200 amino acid BAH domain has been identified in the N-termini of Orc1 orthologues from yeast to humans and in other proteins involved in chromatin structure. The yeast Sir3 BAH domain interacts directly with nucleosomes, suggesting a fundamental role in chromatin organization [25]. The role of the BAH domain of Orc1 was investigated by comparing wild-type ORC1 and orc1bahΔ cells. Biochemical analyses revealed that the Orc1BAH domain was important for the stable association of the ORC with yeast chromatin. Chromatin immunoprecipitation with Orc1 antibodies followed by microarray analyses showed that the Orc1BAH domain made a substantial contribution to a group of origins known as 'orc1bahΔ-sensitive origins'. Enriched ORC peaks were both narrower and smaller in the mutant than in the wild type. 2D electrophoresis experiments for determining the efficiency of origin firing showed that *orc1bah*Δ-sensitive ARSs were more strongly affected than *orc1bah*Δ-resistant ARSs in the mutant. Finally, NFRs containing *orc1bah*Δ-sensitive ARSs are smaller than NFRs containing *orc1bah*Δ-resistant ARSs. This smaller size is at least partly due to a nucleosome at the 5′-end of these origins encroaching on the ORC binding site (Figure 1A). This suggests that, in *orc1bah*Δ-sensitive origins, the nucleosome positioned 5′ to the NFR interacts directly or indirectly with the Orc1BAH domain and promotes ORC-origin association. These data support a model in which ORC recognizes a composite binding site within *orc1bah*Δ-sensitive origins consisting of both a nucleosome and an ACS. It also suggests that functional ACS may be subject to diverse regulation in the yeast genome. The importance of the Orc1BAH domain for origin selection in metazoans has yet to be studied in detail, but yeast *orc1bah*Δ-sensitive origins may turn out to be good models for metazoan origins. NFRs and sites of rapid nucleosome turnover were also shown to be preferential ORC binding sites in a genome-wide study in *Drosophila melanogaster* [26, 27]. It is intriguing that, for both the yeast and fly genomes, models based on physical properties of the DNA are able to identify NFRs in promoter regions, whereas such models are unable to identify NFRs in mammalian genomes [28].

IMPACT OF NUCLEOSOME ORGANIZATION ON REPLICATION INITIATION IN VERTEBRATES

Identification of the molecular mechanisms responsible for origin selection in mammals has been hampered by a lack of a comprehensive mapping of origins of replication within the genome and by problems establishing a powerful genetic model system for the accurate and easy detection of origin activity. Attempts to map Pre-RC complexes in mammals have been unsuccessful, mostly due to a lack of significant enrichment over background in immunoprecipitated material [29]. An alternative way of mapping origins involves the trapping of small replication bubbles, which exist only transiently and are therefore difficult to purify. Two large-scale studies based on the purification of short nascent strands (SNS) and their hybridization to DNA microarrays have explored about 1% of the human and mouse genomes [10, 14]. These studies detected sites of replication initiation via at least two important regulated processes—the formation of Pre-RCs and their activation in S phase—providing a global view of replication initiation in a large cell population. The results obtained in these two studies strongly suggest a role of functional transcriptional elements, such as CpG islands (CGI), promoters and enhancers, in the regulation of DNA replication initiation.

Based on the role of intrinsic histone-DNA preferences in determining *in vivo* nucleosome occupancy in yeast, a recent study explored the possibility that a similar system operates in the human genome [30]. Comparison of the *in vitro* nucleosome positioning signals obtained with yeast genomic DNA and

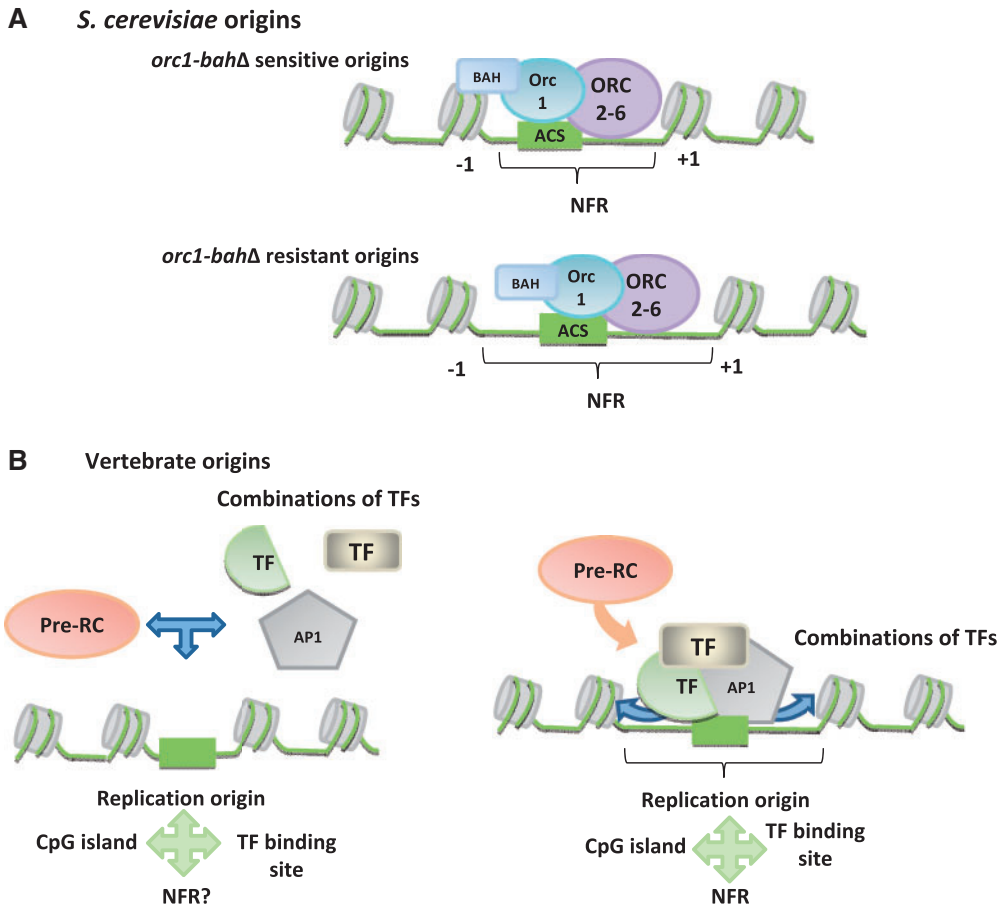

Figure I: Multifactorial regulation of origin specification. (**A**) In *S. cerevisiae*, two classes of origin have been described, according to sensitivity to the BAH domain of the Orc1 subunit. All origins possess a conserved motif, ACS, which is bound by the ORC complex, and this ACS motif specifies a low level of nucleosome occupancy, creating a permissive environment for ORC binding. At *orc1-bah*Δ-sensitive origins, the Orc1-BAH domain interacts directly or indirectly with the nucleosome in position −1, affecting its positioning, thereby reducing the size of the NFR (This figure is adapted from [24]). (**B**) Regulation of metazoan origins is less well understood, but several factors have been identified as probably or possibly involved in regulating origin specification. In *cis*, a replication origin may be determined by several elements, such as CGI, NFRs or TF binding sites. These elements are often co-localized or influence each other, blurring identification of the determinant feature for origin specification. For replication initiation, the Pre-RC complex must be recruited to the origin, but the exact mechanism underlying this recruitment remains unclear. It has been suggested that the binding of a combination of TFs might lead to chromatin being organized into a structure permissive for Pre-RC recruitment. Direct or indirect contacts between TFs and Pre-RC subunits may also contribute to origin selection.

in vivo maps of nucleosomes in human cells showed that there was a significant relationship between intrinsic and *in vivo* nucleosome occupancy, but that intrinsic occupancy accounted for only a fraction of *in vivo* nucleosome occupancy [31]. Examination of the intrinsic nucleosome occupancy of human regulatory elements, including promoters and non-promoter regions associated with DNaseI-hypersensitive sites, showed that, in all cases, these regions displayed higher than average intrinsic nucleosome occupancy. Moreover, in nearly all cases, these regions also displayed higher than average *in vivo* nucleosome occupancy, rather than the lower levels of occupancy observed in yeast. The exception to the overall correlation between intrinsic and *in vivo* nucleosome occupancy in regulatory regions was the strong nucleosome depletions just upstream from the transcription start site (TSS) in CGI promoters *in vivo*. This discrepancy may be due to the binding of *trans*-acting factors (Figure 1B). However, the authors noted that, on average, poly-A content increases at precisely the position of CGI promoters, raising the possibility that depletion may be at least partly due to intrinsic nucleosome

sequence preferences not incorporated into the imperfect model. An *in vitro* nucleosome assembly map of the human genome would help to resolve this issue and to refine the model. One hypothesis for the high degree of occupancy of regulatory elements in the human genome is that such a constraint would tend to reinforce co-operative interactions between transcription factors (TFs) in displacing nucleosomes, potentially providing an additional level of specificity in gene regulation. The truly intrinsic properties of CGI promoters in terms of nucleosome organization remain to be investigated. This is of particular importance for origin selection, because ~50% of the origins found in 1% of the human genome are located at or near a CGI. If human CGIs do indeed encode NFRs, it may be that, as in yeast, ORC complexes are preferentially assembled at NFRs. Future developments in both origin and Pre-RC mapping throughout the entire human genome and the *in vitro* reconstitution of nucleosomes with the human genome should help to resolve the issue of whether a subset of human origins is favoured by the DNA-encoded information about nucleosome exclusion.

Other structural properties of chromatin structure known to increase the accessibility to DNA have also been proposed to regulate the position of origins of replication, by analogy with the regulation of transcription. The idea that histone modifications might control the positioning of replication origins is attractive at first glance, as it would provide a means of adapting origin positioning to the establishment of different cell types. However, experimental data are not in favour of a predominant role of open chromatin marks in origin selection. Several studies have focused on specific loci during cell differentiation, such as the HoxB locus in mouse and the β-globin locus in chicken [32–34]. No clear link has been found between the changes in origin pattern and changes in histone modification at either of these loci. In particular, the acetylation of H3 and H4 has been shown to be dispensable for origin activation. Similarly, the same origins are used on the active and inactive X chromosomes, which have different chromatin structures [35]. A large-scale study in human HeLa cells also analysed the correlation between origin positioning and histone marks along ENCODE regions [10]. Only a small fraction of the genome was covered by this dataset (1%), but it should be possible to generalize the conclusions drawn from this analysis because ENCODE regions were 44 discrete regions selected as regions representative of the whole genome. Almost half the 283 origins detected did not overlap with regions of H3 or H4 acetylation or of di- or trimethylation of H3K4. Moreover, CGIs harbouring H3K4me3 are not better substrates for origin specification than CGIs lacking this marker. We cannot rule out the possibility that histone modifications known to be associated with active transcription are important for a subset of origins, but this study demonstrated that a large proportion of origins are not regulated by these canonical histone marks. As only a limited number of histone modification marks were assayed, it remains possible that origins lacking the classical markers of open chromatin have an unexplored modification, perhaps affecting the histone core rather than its tail. This observation could be due to an abnormal regulation of replication origins in HeLa cells, however similar conclusions were reached in mouse embryonic stem (ES) cells [14]. A recent study in hamster cells based on DNA molecular combing also analysed both origin positioning and origin firing frequency as a function of chromatin structure, for the AMPD2 (adenosine monophosphate deaminase 2) locus [36]. Origin efficiency is the fraction of cells in which a particular origin is used during S phase. It varies between origins and may vary, for a particular origin, in response to stress or during differentiation [37, 38]. The power of single-fibre analysis lies in its ability to measure origin efficiency precisely. These authors previously showed that the speed of replication fork progression influences origin usage in the amplified 180 kb domain [39]. In conditions of slow replication dynamics, initiation events occur at high density and are distributed evenly between different origins of the locus, whereas in conditions of rapid replication, 70% of initiation events occur at a single origin, oriGNAI3. The authors showed that both efficient and inefficient origins may be associated with acetylated and with non-acetylated H3 or H4 histones. Moreover, changes in origin hierarchy induced by changes in replication dynamics are not correlated with local changes in histone acetylation. As origin licensing takes place during G1, the authors also analysed patterns of histone acetylation in cells arrested in G1. They detected only slight differences in acetylation levels between cells arrested in G1 and exponentially growing cells. These results, obtained with single-molecule approaches to the mapping of origins and the quantification of origin efficiency,

confirm that local histone acetylation is not a key regulator of origin selection in mammals, in contrast to what has been reported for transcriptional activity. In conclusion, the search for critical histone marks involved in replication origin selection in mammals has been unsuccessful, but has demonstrated that origin selection is more complex than previously thought and that new avenues should be explored.

POTENTIAL DIRECTIONS FOR FUTURE INVESTIGATIONS

Now that some of the prime suspects have been eliminated from the investigation, the search for critical regulatory mechanisms involved in origin selection remains an open question to be addressed in higher eukaryotes. It should be borne in mind that, although genome-wide studies in yeast provide compelling evidence that NFRs are important for origin selection, the yeast ORC complex also recognizes a discrete ACS motif, adding an important extra layer of regulation to the definition of replication origins. How can studies at the whole-genome scale help us determine whether specific DNA motifs are also key regulators of origin selection in higher eukaryotes? The strong overlap between transcriptional regulatory elements and replication origins suggests that TFs may be involved in this regulation [10, 14]. TFs may affect replication initiation via two mechanisms: their binding can destabilize nucleosomes, thereby creating a favourable environment for Pre-RC assembly (Figure 1B). Alternatively, direct or indirect protein–protein interactions between TFs and replication factors may also be involved (Figure 1B). So, what would be the best way to investigate such connections? A combination of the increasingly commonly used deep-sequencing methods and powerful statistical analyses should make it possible to map origins for entire genomes therefore allowing to explore all types of chromosomal landscape including regions containing many repeats. Moreover, the collection of data for different cell types and at different stages of differentiation should provide important insight into the coupling of DNA replication to the establishment of expression programmes. The recent profusion of ChIP-sequencing data for several TFs should make it possible to test many factors and their potential correlation with origin positioning. This approach has already been applied to some regions of the human genome, leading to the conclusion that the c-Jun and c-Fos TFs (forming the AP-1 complex) are significantly associated with 20% of the origins mapped and that this strong association could not be attributed solely to the coincidence of origins and CGIs [10]. These findings suggest that the AP-1 complex may be a key regulator of replication initiation for a subset of origins. Further large-scale data sets for replication origin mapping might also facilitate the search for *de novo* motifs. The anticipated composite and variable organization of replication origins is a major obstacle to such studies, but at least we now have the possibility of exploring this alternative way of working. Finally, we also need to develop new, powerful genetic tools for dissection of the molecular events occurring at specific strong origins. This would make it possible to test new hypotheses developed through genome-wide studies and to improve our understanding of the mechanisms of origin specification.

Key Points

- Recent genome-wide analyses have provided insight into the regulation of replication origin selection in both yeast and vertebrates.
- It has become increasingly clear that open chromatin marks, such as histone acetylation, cannot account for origin specification. However, NFRs have been implicated in origin specification in yeast and in fly, but further studies are required to explore this link in vertebrates.
- We suggest here that TFs may help to recruit the Pre-RC to origins, either by direct interaction or via the displacement of nucleosomes.

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